The role of salicylic acid in response of two rice cultivars to chilling stress

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Abstract

Two rice (*Oryza sativa* L.) cultivars differing in chilling sensitivity, Changbaijiu (chilling-tolerant) and Zhongjian (chilling-sensitive) were pre-treated with 0.5, 1.0 and 2.0 mM salicylic acid (SA) for 24 h before chilling at 5 °C for 1 d. Chilling induced SA accumulation, particularly conjugated SA in both leaves and roots of the two rice cultivars. After SA administration, SA accumulated in the roots of both cultivars at a concentration-dependent manner, whereas only a slight increase was observed in their leaves. Conjugated SA accounted for most of the increase. The beneficial effect of SA treatment on protecting rice seedlings from chilling injury was not observed at any concentration in either cultivar. Pre-treatment with SA even decreased their chilling tolerance confirmed by increased electrolyte leakage and lipid peroxidation. Further, most of the activities of antioxidant enzymes decreased or remained unchanged in leaves and roots of SA pre-treated seedlings after chilling. These results implied that down-regulation of antioxidant defence might be involved in the reduction of chilling tolerance in SA-pre-treated plants.

Additional key words: antioxidant enzymes, lipid peroxidation, *Oryza sativa* L.

Introduction

Salicylic acid (SA) is a natural phenolic compound and endogenous signal involved in plant defence responses as well as the regulation of plant growth and development (Raskin 1992, Dempsey *et al.* 1999). During recent years, SA has received particular attention because of its role in modulating plant response to several abiotic stresses, such as chilling, heat, drought, salt and ultraviolet radiation. It was reported that SA ameliorated the damaging effects of heavy metals in rice (Mishra and Choudhuri 1999, Guo *et al*. 2007a) and NaCl stress in tomato (He and Zhu 2008), improved the heat-shock tolerance of mustard and tobacco plants (Dat *et al.* 1998a, 2000), increased chilling tolerance of maize, cucumber, wheat, bean, tomato and banana (Janda *et al*. 1999, Senaratna *et al*. 2000, Ding *et al*. 2002, Kang and Saltveit 2002, Kang *et al*. 2003, Tasgin *et al*. 2003). However, reverse reports also exist. Pre-treatment with 0.5 mM SA decreased drought tolerance in maize (Németh *et al*. 2002) and spring wheat (Horváth *et al*. 2007) and reduced freezing tolerance of winter wheat (Horváth *et al*. 2007). Further, SA-deficient *Arabidopsis* expressing *NahG* gene exhibited higher tolerance to NaCl and osmotic stress than wild-type plants (Borsani *et al*. 2001). Apparently, SA has broad but divergent effects on stress acclimation or damage development of plants (Metwally *et al*. 2003).

Among all plants surveyed, rice leaves have the highest endogenous SA content (Raskin *et al*. 1990, Silverman *et al*. 1995), but rice roots have very low contents of SA (Chen *et al*. 1997). In contrast to tobacco, cucumber and *Arabidopsis*, in which endogenous contents of SA and its conjugates increase immediately after pathogen infection (Malamy *et al*. 1990, Métraux *et al*. 1990, Rasmussen *et al*. 1991, Uknes *et al*. 1993), little or no change in SA content is found in rice seed-lings after inoculation with

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Received 17 November 2008, *accepted* 20 July 2009.
Abbreviations: APX - ascorbate peroxidase; AsA - ascorbic acid; CAT - catalase; GPX - guaiacol peroxidase; GR - glutathione reductase; PR - pathogenesis-related; PVPP - polyvinylpolypyrrolidone; SA - salicylic acid; SOD - superoxide dismutase; TBA - thiobarbituric acid; TBARS - thiobarbituric acid reactive substances.
Acknowledgements: We extend our sincere thanks to the Chinese Rice Research Institute and the Rice Research Institute of Jilin Academy of Agricultural Sciences for their kind donation of rice seeds. This study was supported by the Program for Key International Sciences and Technology Cooperation Project of China (2001CB711103).
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bacterial or fungal pathogens (Silverman *et al*. 1995). On the other hand, although exogenous application of SA or its analogues activate *PR* gene expression and systemic acquired resistance in tobacco and *Arabidopsis* (Ryals *et al*. 1996), SA is a poor activator of *PR* gene expression in rice and can not induce disease resistance. Thus, based on their data and previous studies in rice, tobacco, *Arabidopsis*, and other plants, Yang *et al*. (2004) divided the plants into SA-sensitive and SA-insensitive species. They proposed that in SA-insensitive plants such as rice, SA may play an important role on modulating redox balance and protect plants from oxidative damage caused by various biotic and abiotic factors, but cannot be an effective secondary signal for activation of defence genes and induced resistance.

Rice plants are prone to be injured at seedling stage when they are grown in early spring in temperate or subtropical environments. Low-temperature effects on rice seedlings can be manifested as poor germination, slow growth, discoloration or yellowing, withering after transplanting, reduced tillering, and stunted growth (Kaneda and Beachell 1974). In order to further investigate the role of SA on protecting rice seedlings from chilling injury occurred at the three leaf stage, the experiments were conducted on two rice cultivars with different chilling sensitivity. The objective of this study was *1*) to determine whether chilling exposure could induce SA accumulations in rice plants, *2*) to examine the effects of exogenous SA application on the endogenous SA levels, *3*) to compare the effects of SA on inducing chilling tolerance in the two rice cultivars with different chilling sensitivity, and *4*) to investigate the role of SA on modulating redox balance through activation of antioxidant enzymes under chilling stress.

Materials and methods

Plants and growth conditions: Sterilized seeds of rice (*Oryza sativa* L.) cv. Changbaijiu (chilling-tolerant) and cv. Zhongjian (chilling-sensitive) were germinated at 32 °C for 48 h in the dark, then, the seedlings were grown in a nutrient solution in a growth chamber (*Sanyo MLR-350HT*, Tokyo, Japan) with a 16-h photoperiod, day/night temperature of 27/22 °C, irradiance of 250 μmol m^{-2} s^{-1} and relative humidity of 80 % as previously described by Makino *et al.* (1988).

Uniform seedlings at the three-leaf stage were selected for the experiments. Some of them were pre-treated with different concentrations (0.5, 1.0, 2.0 mM) of SA (*Fluka*, USA) which were added to the solution for 24 h. Then, SA-pre-treated plants and control plants were chilled at 5 °C for 24 h under the same irradiance and photoperiod. The third leaves and roots of the seedlings were harvested for chilling injury determination, SA analysis and antioxidant enzyme assay.

Measurement of electrolyte leakage and lipid peroxidation: The electrolyte leakage was determined according to the method described by Guo *et al*. (2007b) with some modifications. Rice leaves or roots were cut into 1 cm segments and immersed in 9 cm^3 deionized water and after 20 min the initial conductivity was determined using a conductivity meter (*Hanna EC215*, Padova, Italy). The conductivity was determined again after the tubes were placed in boiling water for 5 min, and cooled to room temperature. The relative electrolyte leakage was calculated as the ratio of conductivity before boiling to that after boiling.

Lipid peroxidation was estimated by measuring the amount of thiobarbituric acid reactive substances (TBARS) according to the method described by Heath and Packer (1968).

Analysis of SA: Free and conjugated SA were determined according to the method of Palva *et al*. (1994) and Li *et al*. (1997) with some modifications. Rice roots or leaves were ground into a fine powder with liquid nitrogen. Then 0.5 g of the leaf or root powder was homogenized with 3.5 cm^3 pre-cooled 80 % ethanol. The extracts were centrifuged at 10 000 *g* for 10 min. The pellet was resuspended in 2 cm^3 of 80 % ethanol and reextracted at 10 000 *g* for another 10 min. The supernatants from both extractions were combined and evaporated to one-tenth of the original volume in a rotary evaporator (*Eyela N-1001*, Tokyo, Japan) at 45 °C. The remnant was diluted with 4 cm^3 2 % (m/v) meta-phosphoric acid and then partitioned three times with 2 cm^3 ethyl acetate. The top organic phases containing free SA were pooled and dried under nitrogen. The remaining aqueous phases containing conjugated SA were adjusted to 1 M HCl and heated at 80 °C in a sealed tube for 1 h to release free SA from acid-labile conjugated forms. The released free SA was then partitioned as described above. Extracts were then redissolved in 4 cm^3 70 % methanol and passed through a *Supelclean ENVI-18* cartridge (*Supelco*, Bellefonte, USA). The eluted fraction was dried in a rotary evaporator (*Eyela N-1001*) at 45 °C and redissolved in 0.2 cm^3 of HPLC mobile phase (45 % methanol solution containing 0.02 % H_3PO_4). Samples were analyzed on a high-performance liquid chromatograph (*Dionex*, Sunnyvale, USA) equipped with a *P680* HPLC Pump, thermostated column compartment *TCC-100* and a photodiode array *PAD-100* detector. A 0.01 cm^3 aliquot per sample was injected into a 5 μm *Kromasil* (Bohus, Sweden) *100-5C18* column (250 × 4.6 mm i.d.) run at 40 °C with an isocratic flow rate of 0.6 cm^3 min^{-1}. The detection wavelengths were 205 and 310 nm.

Assays of antioxidant enzymes: Both the leaves and roots were harvested and ground using liquid nitrogen. The 0.3 g of powder was homogenized under ice-cold conditions in 1.5 cm^3 reaction mixture composed of 100 mM phosphate buffer (pH 7.6), 1.0 mM EDTA, 0.3 % (v/v) *Triton X-100*, 1 % (m/v) PVPP and 1 mM ascorbic acid (AsA). The homogenate was centrifuged at 12 000 *g* for 20 min. The supernatant was used for enzyme assays.

Ascorbate peroxidase (APX; EC 1.11.1.11) was assayed by monitoring the oxidation of AsA at 290 nm (coefficient of absorbance, $\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$) according to the method of Nakano and Asada (1981). Catalase (CAT; EC 1.11.1.6) activity was assayed as a decrease of absorbance at 240 nm ($\varepsilon = 0.04\ mM^{-1}\ cm^{-1}$) due to the consumption of H_2O_2, by the method of Knörzer *et al*. (1996). The activity of glutathione reductase (GR; EC 1.6.4.2) was determined according to the method of Grace and Logan (1996). The decrease in absorbance at 340 nm ($\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$), as a result of oxidation of NADPH, was determined. Guaiacol peroxidase (GPX; EC 1.11.1.7) activity was determined as the increase in absorbance at 470 nm following the formulation of tetraguaiacol ($\varepsilon = 26.6\ mM^{-1}\ cm^{-1}$), according to the method of Britton and Maehly (1955). Superoxide dismutase (SOD; EC 1.15.1.1) activity assay was based on the method of Beyer and Fridovich (1987). The photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm was measured and 1 unit of SOD activity is defined as the amount of enzyme required inhibiting the photoreduction of NBT by 50 %. Protein content was determined according to the method of Bradford (1976) using BSA as a standard.

Statistical analysis: All the data presented here are based on two independent experiments with similar results. Each experiment was conducted with three different pots for the same treatment. Values presented were mean ± SE for three replicates. To assess the statistical significance of treatment differences, a one-way analysis of variance (*ANOVA*) followed by the least significant difference (LSD) comparison at different levels was employed.

Results

After 1 d of chilling at 5 °C, Zhongjian seedlings seemed more withered than Changbaijiu seedlings. The electrolyte leakage in both leaves and roots of Zhongjian also increased to a greater extent (385 and 41 %) after chilling exposure than in Changbaijiu (277 and 15 %), suggesting more severe cold injury occurred in chilling-sensitive rice cultivar (Table 1). This observation was confirmed by lipid peroxidation estimations. The TBARS content increased markedly in Zhongjian leaves whereas it remained unchanged in Changbaijiu leaves (Table 1).

Generally, there are no obvious differences in endogenous SA contents between the two rice cultivars despite their different chilling sensitivity (Table 1). Under normal growth conditions, low contents of free and conjugated SA were observed in roots of both rice cultivars, but their leaves contained more SA, especially its free forms. After chilling at 5 °C for 24 h, both free and conjugated SA contents increased significantly in roots and leaves of either cultivar except that free SA content remained unchanged in leaves of Changbaijiu (Table 1). The conjugated SA increased to a greater extent than the free forms in both roots and leaves of each cultivar. The magnitude of SA accumulation in Zhongjian seedlings was higher than that in Changbaijiu seedlings.

In the roots, both free and conjugated SA levels increased dramatically after SA pre-treatment in a dose-dependent manner in either cultivar (Table 2). However, in the case of leaves, for Changbaijiu, SA application had almost no effect on free SA content but increased conjugated SA contents by 72, 23 and 34 % at concen-

Table 1. Relative electrolyte leakage, TBARS, free SA and conjugated SA contents in leaves and roots of Changbaijiu and Zhongjian under control (27/22 °C) and chilling conditions (24 h at 5 °C). Means ± SE, $n = 3$. *Asterisks* indicate significant differences from controls (* - $P < 0.05$, ** - $P < 0.01$, *** - $P < 0.001$).

Parameters	Treatments	Leaf		Root	
		Changbaijiu	Zhongjian	Changbaijiu	Zhongjian
Relative electrolyte leakage [%]	control	2.46 ± 0.12	2.83 ± 0.42	17.35 ± 0.43	8.97 ± 0.95
	chilling	7.43 ± 1.47*	13.75 ± 0.32**	20.01 ± 0.94	12.66 ± 0.23*
TBARS content [nmol g^{-1}(d.m.)]	control	33.67 ± 1.48	23.40 ± 2.50	54.11 ± 1.07	37.79 ± 1.49
	chilling	29.58 ± 3.97	34.92 ± 7.12*	49.45 ± 2.55	34.99 ± 7.87
Free SA content [μg g^{-1}(d.m.)]	control	15.56 ± 0.31	12.98 ± 0.43	2.82 ± 0.46	4.48 ± 0.68
	chilling	13.67 ± 0.96	17.42 ± 0.25***	6.60 ± 0.76*	8.03 ± 0.83*
Conjugated SA content [μg g^{-1}(d.m.)]	control	9.37 ± 0.22	5.53 ± 0.33	2.94 ± 0.44	3.95 ± 0.27
	chilling	17.61 ± 1.53**	25.40 ± 1.90*	9.34 ± 1.38*	23.73 ± 5.95*

Table 2. Endogenous SA content [μg g^{-1} (f.m.)] in leaves and roots of Changbaijiu and Zhongjian after pre-treatment with different concentrations of SA for 24 h. Means ± SE, n = 3. *Asterisks* indicate significant differences from controls (* - $P < 0.05$, ** - $P < 0.01$, *** - $P < 0.001$).

Cultivar	SA [mM]	Leaf free SA	conjugated SA	total SA	Root free SA	conjugated SA	total SA
Changbaijiu	0.0	2.96 ± 0.06	1.78 ± 0.04	4.75 ± 0.10	0.25 ± 0.04	0.27 ± 0.04	0.52 ± 0.08
	0.5	3.06 ± 0.15	3.16 ± 0.03***	6.21 ± 0.16**	3.13 ± 0.08***	34.92 ± 1.12**	38.05 ± 1.11***
	1.0	2.76 ± 0.23	2.24 ± 0.08**	5.00 ± 0.18	3.24 ± 0.20***	148.09 ± 5.90***	151.34 ± 5.90***
	2.0	2.83 ± 0.21	2.38 ± 0.07***	5.22 ± 0.28	5.15 ± 0.16***	163.08 ± 5.17***	168.23 ± 5.33***
Zhongjian	0.0	2.85 ± 0.09	1.21 ± 0.07	4.06 ± 0.13	0.28 ± 0.04	0.25 ± 0.02	0.53 ± 0.05
	0.5	3.38 ± 0.17	1.47 ± 0.11	4.82 ± 0.31	2.44 ± 0.27	118.50 ± 4.74***	117.28 ± 5.96***
	1.0	2.87 ± 0.10	3.45 ± 0.25***	6.32 ± 0.26**	2.72 ± 0.06*	214.12 ± 4.50***	216.84 ± 4.54***
	2.0	6.73 ± 0.28***	3.52 ± 0.13***	10.25 ± 0.38***	16.94 ± 0.98***	253.44 ± 0.15***	271.20 ± 0.91***

Table 3. The effects of SA pre-treatment on relative electrolyte leakage [%] and TBARS content [nmol g^{-1} (d.m.)] in leaves and roots of Changbaijiu and Zhongjian under chilling conditions. Means ± SE, n = 3. *Asterisks* indicate significant differences from controls (* - $P < 0.05$, ** - $P < 0.01$, *** - $P < 0.001$).

Cultivar	SA [mM]	Leaf electrolyte leakage	TBARS content	Root electrolyte leakage	TBARS content
Changbaijiu	0.0	7.43 ± 1.47	29.58 ± 3.97	20.01 ± 0.94	49.45 ± 2.55
	0.5	12.51 ± 0.44	31.28 ± 4.00	27.74 ± 0.61*	53.00 ± 2.22
	1.0	23.30 ± 6.40*	40.85 ± 4.04	29.69 ± 1.84**	53.32 ± 5.37
	2.0	37.87 ± 3.46**	39.18 ± 2.51	31.70 ± 3.15**	58.78 ± 4.39
Zhongjian	0.0	13.75 ± 0.32	34.92 ± 3.56	12.66 ± 0.23	34.99 ± 7.87
	0.5	28.24 ± 4.17*	36.44 ± 0.48	18.95 ± 1.53	51.03 ± 2.77
	1.0	46.28 ± 0.66***	38.09 ± 1.86	20.22 ± 0.94*	48.36 ± 4.13
	2.0	53.23 ± 1.38***	42.55 ± 1.70*	22.42 ± 1.28**	48.55 ± 4.76

trations of 0.5, 1.0 and 2.0 mM, respectively. The same held true for Zhongjian leaves. No major changes in the content of free SA occurred at 0.5 mM and 1.0 mM SA, whereas 2.0 mM SA enhanced free SA content by 136 % as compared with the control plants. The content of conjugated SA in Zhongjian leaves also increased with the concentrations of SA applied.

It seemed that SA pre-treatment at any concentration had no effects on alleviating chilling injury symptoms in the rice cultivars used in this experiment. Both Changbaijiu and Zhongjian seedlings pre-treated with SA were even more withered as compared with their respective control plants under chilling conditions. After being chilled at 5 °C for 24 h, the relative electrolyte leakage increased significantly in both leaves and roots of either cultivar pre-treated with SA (Table 3). It seemed that SA pre-treatment elevated electrolyte leakage in a concentration-dependent manner under chilling conditions. However, under normal growth conditions, SA did not affect the values of relative electrolyte leakage in rice seedlings at any concentration (data not shown). Further, after being pre-treated with SA, Zhongjian seedlings withered more rapidly than Changbaijiu seedlings when exposed to chilling.

After application of different concentrations of SA, both the leaves and roots of each cultivar exhibited an uptrend tendency in TBARS content, however, statistically significant increase in TBARS content was observed only in the Zhongjian leaves pre-treated with 2.0 mM SA under chilling (Table 3).

At normal growth conditions, the activities of CAT and GR increased in a dose-dependent manner, while the activities of GPX and SOD remained unchanged after SA treatment in roots of both cultivars (Table 4). In addition, a dramatic increase in APX activity was observed in roots of Changbaijiu pre-treated with 2.0 mM SA, but not in roots of Zhongjian (Table 4). In the case of leaves, pre-treatment with SA had no effect on the activities of GPX, GR and SOD in either cultivar at any concentration, but SA pre-treatment elevated CAT activity in leaves of Changbaijiu by 19 % at the concentration of 2.0 mM, and reduced APX activity in leaves of Zhongjian by 23 and 16 % at the concentration of 0.5 and 1.0 mM, respectively. The activity of APX in Changbaijiu leaves and that of CAT in Zhongjian leaves remained unchanged after SA pre-treatment.

Table 4. The effects of SA pre-treatment on APX, CAT, GPX, GR and SOD activities in leaves and roots of Changbaijiu and Zhongjian under normal growth conditions. Means ± SE, $n = 3$. *Asterisks* indicate significant differences from controls (* - $P < 0.05$, ** - $P < 0.01$, *** - $P < 0.001$).

	SA [mM]	Leaf Changbaijiu	Zhongjian	Root Changbaijiu	Zhongjian
APX activity	0.0	0.28 ± 0.03	0.42 ± 0.02	1.44 ± 0.08	2.57 ± 0.09
[μmol mg^{-1} (protein) min^{-1}]	0.5	0.29 ± 0.02	0.32 ± 0.12*	1.57 ± 0.06	2.59 ± 0.05
	1.0	0.26 ± 0.01	0.35 ± 0.01*	1.66 ± 0.07	2.59 ± 0.15
	2.0	0.27 ± 0.01	0.38 ± 0.02	1.82 ± 0.09**	2.49 ± 0.07
CAT activity	0.0	12.49 ± 0.72	19.37 ± 0.38	13.63 ± 0.96	60.61 ± 0.84
[μmol mg^{-1} (protein) min^{-1}]	0.5	12.55 ± 0.15	18.52 ± 0.77	12.68 ± 0.63	67.60 ± 1.55*
	1.0	11.98 ± 0.31	17.95 ± 1.04	13.42 ± 0.47	65.59 ± 2.04
	2.0	14.81 ± 0.19**	18.74 ± 0.29	21.58 ± 0.37***	72.40 ± 0.97**
GPX activity	0.0	0.16 ± 0.01	0.56 ± 0.03	3.93 ± 0.12	10.69 ± 0.74
[μmol mg^{-1} (protein) min^{-1}]	0.5	0.17 ± 0.01	0.57 ± 0.01	4.39 ± 0.34	11.56 ± 0.51
	1.0	0.15 ± 0.01	0.55 ± 0.03	4.39 ± 0.29	9.71 ± 0.41
	2.0	0.18 ± 0.01	0.62 ± 0.02	3.88 ± 0.28	10.48 ± 0.49
GR activity	0.0	62.64 ± 1.90	74.23 ± 3.91	132.02 ± 8.57	97.46 ± 1.56
[nmol mg^{-1} (protein) min^{-1}]	0.5	65.48 ± 1.55	74.83 ± 7.96	147.31 ± 12.3	127.70 ± 3.43
	1.0	59.96 ± 1.20	64.96 ± 1.63	169.60 ± 4.75*	174.19 ± 11.1**
	2.0	69.09 ± 2.82	61.99 ± 6.63	181.57 ± 1.79**	175.56 ± 16.3**
SOD activity	0.0	6.53 ± 0.28	4.98 ± 0.14	18.43 ± 2.83	6.66 ± 0.86
[unit mg^{-1} (protein)]	0.5	6.76 ± 0.33	5.20 ± 0.60	21.31 ± 0.58	7.23 ± 1.89
	1.0	6.28 ± 0.31	4.61 ± 0.33	20.31 ± 2.00	5.72 ± 0.27
	2.0	5.69 ± 0.27	5.15 ± 0.28	16.15 ± 1.23	6.45 ± 0.36

Table 5. The effects of SA pre-treatment on the APX, CAT, GPX, GR and SOD activities in leaves and roots of Changbaijiu and Zhongjian under chilling conditions. Means ± SE, $n = 3$. *Asterisks* indicate significant differences from controls (* - $P < 0.05$, ** - $P < 0.01$, *** - $P < 0.001$).

	SA [mM]	Leaf Changbaijiu	Zhongjian	Root Changbaijiu	Zhongjian
APX activity	0.0	0.27 ± 0.01	0.39 ± 0.02	2.68 ± 0.11	1.65 ± 0.10
[μmol mg^{-1} (protein) min^{-1}]	0.5	0.25 ± 0.01	0.23 ± 0.01***	2.07 ± 0.07**	1.41 ± 0.09
	1.0	0.23 ± 0.01	0.23 ± 0.01***	2.02 ± 0.06**	1.59 ± 0.07
	2.0	0.28 ± 0.01	0.26 ± 0.02***	2.18 ± 0.06*	1.61 ± 0.04
CAT activity	0.0	8.51 ± 0.35	20.02 ± 0.60	30.29 ± 1.71	67.93 ± 1.42
[μmol mg^{-1} (protein) min^{-1}]	0.5	2.90 ± 0.27***	14.95 ± 1.13**	22.12 ± 0.64**	70.99 ± 2.19
	1.0	3.38 ± 0.29***	13.62 ± 0.31***	20.27 ± 0.78***	68.74 ± 1.56
	2.0	5.46 ± 0.13***	15.47 ± 0.48**	27.40 ± 0.54	70.89 ± 4.03
GPX activity	0.0	0.32 ± 0.01	0.49 ± 0.02	8.11 ± 0.02	10.24 ± 0.31
[μmol mg^{-1} (protein) min^{-1}]	0.5	0.33 ± 0.05	0.42 ± 0.02	7.95 ± 0.91	8.11 ± 0.41**
	1.0	0.32 ± 0.03	0.40 ± 0.02	8.47 ± 1.47	8.89 ± 0.38*
	2.0	0.39 ± 0.01	0.44 ± 0.03	8.66 ± 0.65	8.29 ± 0.33**
GR activity	0.0	63.44 ± 1.10	50.25 ± 2.89	139.14 ± 4.58	138.02 ± 7.90
[nmol mg^{-1} (protein) min^{-1}]	0.5	60.97 ± 1.82	54.06 ± 0.50	142.07 ± 11.33	146.93 ± 11.04
	1.0	71.39 ± 6.20	60.67 ± 2.44	151.38 ± 13.17	150.37 ± 5.93
	2.0	82.85 ± 4.05**	56.84 ± 7.16	163.37 ± 3.35	152.60 ± 5.79
SOD activity	0.0	5.07 ± 0.04	5.69 ± 0.44	16.00 ± 2.19	6.28 ± 0.31
[unit mg^{-1} (protein)]	0.5	4.93 ± 0.21	4.66 ± 0.31	13.35 ± 1.31	7.48 ± 0.63
	1.0	5.02 ± 0.10	4.32 ± 0.05	13.05 ± 1.56	8.73 ± 0.79
	2.0	5.06 ± 0.24	4.45 ± 0.70	10.37 ± 1.43	7.11 ± 1.23

Under chilling conditions, SA pre-treatment reduced the activities of APX and CAT significantly in roots of Changbaijiu, while in Zhongjian roots, GPX activity was reduced remarkably by SA pre-treatment (Table 5). As for GR and SOD, no major change was detected in the roots of either cultivar pre-treated with SA. In the case of SA pre-treated leaves, CAT activity decreased significantly in both cultivars, while APX activity decreased only in Zhongjian leaves. It is worth noting that a 31 % increase in GR activity was observed in leaves of Changbaijiu after 2.0 mM SA pre-treatment.

Discussion

It has been reported that both biotic and abiotic stress can cause SA accumulations in many plant species. After pathogen inoculation, large amounts of SA was accumulated in cucumber, tobacco and *Arabidopsis* (Malamy *et al*. 1990, Métraux *et al*. 1990, Rasmussen *et al*. 1991, Uknes *et al*. 1993). Ozone, low temperature and salinity can also stimulate SA accumulations in tobacco and *Arabidopsis* (Yalpani *et al*. 1994, Scott *et al*. 2004). However, in the case of rice, the situation is somewhat complicated. No major change in SA content was detected following infection by either bacterial or fungal pathogens (Silverman *et al*. 1995, Yang *et al*. 2004), whereas NaCl treatment obviously increased SA contents in rice seedlings as compared with the control (Shim *et al*. 2003, Sawada *et al*. 2006). The present results showed that chilling, like salinity, also significantly induced SA accumulations in both the leaves and roots of the rice plants. Further, conjugated SA accounted for most of the increase, as in other stresses (Yalpani *et al*. 1994, Sharma *et al*. 1996, Chamnongpol *et al*. 1998, Dat *et al*. 1998*b*, Scott *et al*. 2004).

Although free forms of SA were added to hydroponic cultures, conjugated SA rather than free SA increased to a greater extent in the roots of both rice cultivars (Table 3). These results implied that the rice roots efficiently absorbed SA from hydroponic solutions, and quickly converted it to conjugate forms. This might be due to the activation of SA-glucosyltransferase by exogenous SA application (Silverman *et al*. 1995). On the other hand, after SA pre-treatment, large amounts of SA accumulated in the rice roots but not in their leaves. The SA contents in pre-treated roots were 1 to 2 orders of magnitude higher than that in the leaves. Thus, it was likely that SA absorbed by the roots might not be easily transported from the roots to the leaves.

In recent years, the beneficial effects of SA treatment on cold tolerance have been described in maize and wheat (Janda *et al*. 1999, Tasgin *et al*. 2003), bean (Senaratna *et al*. 2000), cucumber (Kang and Saltveit 2002), tomato (Senaratna *et al*. 2000, Ding *et al*. 2002), banana (Kang *et al*. 2003), and Persian lilac (Bernard *et al*. 2002). However, in this study, no obvious alleviation of chilling injury was observed in either rice cultivar after pre-treatment with SA. Moreover, SA pre-treatment even exacerbated the chilling injury occurred at the three leaf stage of the rice seedlings. It has been demonstrated in many plant species that the protection effects caused by SA were due to up-regulation of antioxidant capacity. The results obtained from banana suggested that SA pre-treatment could directly or indirectly activate antioxidant enzymes during chilling stress (Kang *et al*. 2003). Kang and Saltveit (2002) also reported that the chilling tolerance induced by SA in maize and cucumber shoots was associated with increases in the activity of GR and GPX. In the present study, SA pre-treatment enhanced the activities of GR and CAT in both rice roots under normal growth conditions. However, the increments were absent when SA pre-treated plants were exposed to chilling conditions. Namely, the activity of almost all the enzymes declined or remained unchanged. Hence, we suggested that decreased antioxidant defence ability appeared to be involved in the reduction of chilling tolerance in SA pre-treated plants.

Research on *Arabidopsis* SA overproduction mutants showed that elevated SA level increased $O_2^{\bullet -}$ production, leading to programmed cell death and hypersensitivity to ozone (Rao and Davis 1999). More recent studies in rice suggested that excessive accumulation of SA induced by high concentrations of NaCl treatment can interrupt the balance between H_2O_2 generation and scavenging, resulting in oxidative injury (Sawada *et al*. 2006). These results suggested that high levels of SA multiply reactive oxygen species (ROS) generation and potentiate activation of an oxidative burst under stress. Therefore, optimal concentrations of SA are required to achieve maximum induction of defence responses and to minimize the adverse influence of stress (Rao and Davis 1999). Our results that increased chilling injury occurred in rice plants which accumulated extremely high SA contents after SA pre-treatment supported this view. Further, when our results are combined with previous results that exogenous SA failed to induce disease resistance not only in rice plants, but also in potato, another plant species with high endogenous SA content, it is likely that the response pattern of plants with high basal SA content to exogenous SA might be different from that of the plants with relatively low basal SA content.

References

Bernard, F., Shaker-Bazarnov, H., Kaviani, B.: Effects of salicylic acid on cold preservation and cryopreserva-tion of encapsulated embryonic axes of Persian lilac (*Melia azedarach* L.). - Euphytica **123**: 85-88, 2002.

Beyer, W.F., Fridovich, I.: Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. - Anal. Biochem. **161**: 559-566, 1987.

Borsani, O., Valpuesta, V., Botella, M.A.: Evidence for a role of salicylic acid in the oxidative damage generated by NaCl and osmotic stress in *Arabidopsis* seedlings. - Plant Physiol. **126**: 1024-1030, 2001.

Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Britton, C., Maehly, A.C.: Assay of catalase and peroxidase. - Methods Enzymol. **2**: 764-775, 1955.

Chamnongpol, S., Willekens, H., Moeder, W., Langebartels, C., Sandermann, H., Jr., Van Montagu, M., Inzé, D., Van Camp, W.: Defense activation and enhanced pathogen tolerance induced by H_2O_2 in transgenic tobacco. - Proc. nat. Acad. Sci. USA **95**: 5818-5823, 1998.

Chen, Z.X., Iyer, S., Caplan, A., Klessig, D.F., Fan, B.F.: Differential accumulation of salicylic acid and salicylic acid-sensitive catalase in different rice tissues. - Plant Physiol. **114**: 193-201, 1997.

Dat, J.F., Foyer, C.H., Scott, I.M.: Changes in salicylic acid and antioxidants during induced thermotolerance in mustard seedlings. - Plant Physiol. **118**: 1455-1461, 1998b.

Dat, J.F., Lopez-Delgado, H., Foyer, C.H., Scott, I.M.: Parallel changes in H_2O_2 and catalase during thermotolerance induced by salicylic acid or heat acclimation in mustard seedlings. - Plant Physiol. **116**: 1351-1357, 1998a.

Dat, J.F., Lopez-Delgado, H., Foyer, C.H., Scott, I.M.: Effects of salicylic acid on oxidative stress and thermotolerance in tobacco. - J. Plant Physiol. **156**: 659-665, 2000.

Dempsey, D.A., Shah, J., Klessig, D.F.: Salicylic acid and disease resistance in plants. - Crit. Rev. Plant Sci. **18**: 547-575, 1999.

Ding, C.K., Wang, C.Y., Gross, K.C., Smith, D.L.: Jasmonate and salicylate induce the expression of pathogenesis-related-protein genes and increase resistance to chilling injury in tomato fruit. - Planta **214**: 895-901, 2002.

Grace, S.C., Logan, B.A.: Acclimation of foliar antioxidant systems to growth irradiance in three broad-leaved evergreen species. - Plant Physiol. **112**: 1631-1640, 1996.

Guo, B., Liang, Y.C., Zhu, Y.G., Zhao, F.J.: Role of salicylic acid in alleviating cadmium toxicity in rice roots. - J. Plant Nutr. **30**: 427-439, 2007a.

Guo, Z.F., Huang, M.Y., Lu, S.Y., Zhao, Y.Q., Zhong, Q.: Differential response to paraquat induced oxidative stress in two rice cultivars on antioxidants and chlorophyll *a* fluorescence. - Acta Physiol. Plant. **29**: 39-46, 2007b.

He, Y., Zhu, Z.J.: Exogenous salicylic acid alleviates NaCl toxicity and increases antioxidative enzyme activity in *Lycopersicon esculentum*. - Biol. Plant. **52**: 792-795, 2008.

Heath, R.L., Packer, L.: Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. - Arch. Biochem. Biophys. **125**: 189-198, 1968.

Horváth, E., Pál, M., Szalai, G., Páldi, E., Janda, T.: Exogenous 4-hydroxybenzoic acid and salicylic acid modulate the effect of short-term drought and freezing stress on wheat plants. - Biol. Plant **51**: 480-487, 2007.

Janda, T., Szalai, G., Tari, I., Páldi, E.: Hydroponic treatment with salicylic acid decreases the effects of chilling injury in maize (*Zea mays* L.) plants. - Planta **208**: 175-180, 1999.

Kaneda, C., Beachell, H.M.: Response of indica-japonica rice hybrids to low temperatures. - Sabrao J. **6**: 17-32, 1974.

Kang, G.Z., Wang, C.H., Sun, G.C., Wang, Z.X.: Salicylic acid changes activities of H_2O_2-metabolizing enzymes and increases the chilling tolerance of banana seedlings. - Environ. exp. Bot. **50**: 9-15, 2003.

Kang, H.M., Saltveit, M.E.: Chilling tolerance of maize, cucumber and rice seedling leaves and roots are differentially affected by salicylic acid. - Physiol. Plant. **115**: 571-576, 2002.

Knörzer, O.C., Durner, J., Boger, P.: Alterations in the antioxidative system of suspension-cultured soybean cells (*Glycine max*) induced by oxidative stress. - Physiol. Plant. **97**: 388-396, 1996.

Li, Z., Yuan, Y., Li, D.: Determination of salicylate in plant leaves by combination with TLC and HPLC. - Plant Physiol. Commun. **33**: 130-132, 1997.

Makino, A., Mae, T., Ohira, K.: Differences between wheat and rice in the enzymatic properties of ribulose-1,5-bisphosphate carboxylase/oxygenase and the relationship to photosynthetic gas exchange. - Planta **174**: 30-38, 1988.

Malamy, J., Carr, J.P., Klessig, D.F., Raskin, I.: Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. - Science **250**: 1002-1004, 1990.

Métraux, J.P., Signer, H., Ryals, J., Ward, E., Wyss-Benz, M., Gaudin, J., Raschdorf, K., Schmid, E., Blum, W., Inverardi, B.: Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. - Science **250**: 1004-1006, 1990.

Metwally, A., Finkerneier, I., Georgi, M., Dietz, K.J.: Salicylic acid alleviates the cadmium toxicity in barley seedlings. - Plant Physiol. **132**: 272-281, 2003.

Mishra, A., Choudhuri, M.A.: Effects of salicylic acid on heavy metal-induced membrane deterioration mediated by lipoxygenase in rice. - Biol. Plant. **42**: 409-415, 1999.

Németh, M., Janda, T., Horváth, E., Páldi, E., Szalai, G.: Exogenous salicylic acid increases polyamine content but may decrease drought tolerance in maize. - Plant Sci, **162**: 569-574, 2002.

Nakano, Y., Asada, K.: Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. - Plant Cell Physiol. **22**: 867-880, 1981.

Palva, T.K., Hurtig, M., Saindrenan, P., Palva, E.T.: Salicylic acid induced resistance to *Erwinia carotovora* subsp. *carotovora* in tobacco. - Mol. Plant-Microbe Interact. **7**: 356-363, 1994.

Rao, M.V., Davis, K.R.: Ozone-induced cell death occurs via two distinct mechanisms in *Arabidopsis*: the role of salicylic acid. - Plant J. **17**: 603-614, 1999.

Raskin, I.: Role of salicylic-acid in plants. - Annu. Rev. Plant Physiol. Plant mol. Biol. **43**: 439-463, 1992.

Raskin, I., Skubatz, H., Tang, W., Meeuse, B.J.D.: Salicylic acid levels in thermogenic and non-thermogenic plants. - Ann. Bot. **66**: 369-373, 1990.

Rasmussen, J.B., Hammerschmidt, R., Zook, M.N.: Systemic

induction of salicylic acid accumulation in cucumber after inoculation with *Pseudomonas syringae* pv. *syringae*. - Plant Physiol. **97**: 1342-1347, 1991.

Ryals, J.A., Neuenschwander, U.H., Willits, M.G., Molina, A., Steiner, H.Y., Hunt, M.D.: Systemic acquired resistance. - Plant Cell **8**: 1809-1819, 1996.

Sawada, H., Shim, I.S., Usui, K.: Induction of benzoic acid-2-hydroxylase and salicylic acid biosynthesis – modulation by salt stress in rice seedlings. - Plant Sci. **171**: 263-270, 2006.

Scott, I.M., Clarke, S.M., Wood, J.E., Mur, L.A.J.: Salicylate accumulation inhibits growth at chilling temperature in *Arabidopsis*. - Plant Physiol. **135**: 1040-1049, 2004.

Senaratna, T., Touchell, D., Bunn, E., Dixon, K.: Acetyl salicylic acid (aspirin) and salicylic acid induce multiple stress tolerance in bean and tomato plants. - Plant Growth Regul. **30**: 157-161, 2000.

Sharma, Y.K., Leon, J., Raskin, I., Davis, K.R.: Ozone-induced responses in *Arabidopsis thaliana*: the role of salicylic acid in the accumulation of defense-related transcripts and induced resistance. - Proc. nat. Acad. Sci. USA **93**: 5099-5104, 1996.

Shim, I.S., Momose, Y., Yamamoto, A., Kim, D.W., Usui, K.: Inhibition of catalase activity by oxidative stress and its relationship to salicylic acid accumulation in plants. - Plant Growth Regul. **39**: 285-292, 2003.

Silverman, P., Seskar, M., Kanter, D., Schweizer, P., Métraux, J.P., Raskin, I.: Salicylic acid in rice – biosynthesis, conjugation, and possible role. - Plant Physiol. **108**: 633-639, 1995.

Tasgin, E., Atici, O., Nalbantoglu, B.: Effects of salicylic acid and cold on freezing tolerance in winter wheat leaves. - Plant Growth Regul. **41**: 231-236, 2003.

Uknes, S., Winter, A.M., Delaney, T., Vernooij, B., Morse, A., Friedrich, L., Nye, G., Potter, S., Ward, E., Ryals, J.: Biological induction of systemic acquired resistance in *Arabidopsis*. - Mol. Plant-Microbe Interact. **6**: 692-698, 1993.

Yalpani, N., Enyedi, A.J., Leon, J., Raskin, I.: Ultraviolet light and ozone stimulate accumulation of salicyclic acid, pathogenesis-related proteins and virus resistance in tobacco. - Planta **193**: 372-376, 1994.

Yang, Y.N., Qi, M., Mei, C.S.: Endogenous salicylic acid protects rice plants from oxidative damage caused by aging as well as biotic and abiotic stress. - Plant J. **40**: 909-919, 2004.